Relevance of synovial fluid chondroitin sulphate as a biomarker to monitor polo pony joints

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Abstract

Osteoarthritis (OA) of the metacarpophalangeal joint is the most common articular disease in polo ponies leading to early retirement. A biomarker that would discriminate between pathological and physiological changes secondary to exercise could be helpful in OA prevention. The aim of this study was to investigate the effects of polo training on synovial fluid biomarkers of inflammation and cartilage turnover in polo ponies of different skill levels. Synovial fluid samples were collected from metacarpophalangeal joints of polo ponies before and during the polo season (320 d). Nucleated cells, soluble protein, prostaglandin E_2 (PGE_2), glycosaminoglycans (GAG), and urea were measured. The main synovial fluid GAG are chondroitin sulphate (CS, ~25 μg/mL) and hyaluronic acid (HA, ~400 μg/mL). After a polo match, a transitory increase in protein and PGE_2, but not CS and HA, occurred (expressed as urea ratio), returning to basal levels in 24 h. During the polo season, the number of synovial fluid nucleated cells was always in the normal range. Increases in protein and HA occurred during the initial 40 to 80 d, returning to basal levels afterwards. In contrast, in polo prospects the concentration of CS steadily increased during the season. Long-term follow-up revealed that the synovial fluid CS was significantly higher in polo ponies that developed joint diseases within 24 months following our study. In conclusion, CS seems to be an early marker of articular cartilage damage.

Résumé

L'arthrose (OA) de l'articulation métacarpophalangienne est la maladie articulaire la plus fréquente chez les poneys de polo menant à la retraite anticipée. Un biomarqueur qui était discriminateur entre les changements pathologiques et physiologiques secondaires au exercice pourrait être utile pour la prévention de l'OA. L'objectif de la présente étude était examiner les effets de l'activité de polo sur les biomarqueurs de l'inflammation et de le métabolisme de la cartilage dans le liquide synovial des poneys de polo de différents niveaux de qualification. Le SF était obtenu à partir de les articulations métacarpophalangiennes de poneys de polo, avant et pendant la saison de polo (320 jours). Les cellules nucléés, protéine soluble, prostaglandine E_2 (PGE_2), glycosaminoglycanes (GAG) et l'urée ont été mesurés. Les principaux GAG de le liquide synovial sont le chondroïtine sulfate (CS, ~25 μg/mL) et l'acide hyaluronique (HA, ~400 μg/mL). Après un match de polo, ocorru une augmentation transitoire de la protéine et de la PGE_2, mais pas de CS et de HA (exprimé comme le raison d'urée), qui a retourné aux niveaux basal dans 24 h. Pendant la saison de polo, le numero de cellules nucléés dans le liquide synovial était toujours normaux. La protéine et le HA augmentaient pendant les premiers 40–80 jours, mais tous les deux sont retournés aux niveaux de base plus tard. En contraste, dans le group de jeunes poneys (G1), la concentration de CS a augmenté régulièrement pendant la saison. Accompagnant à long terme avait révéle que le CS de liquide synovial était significativement plus élevée chez les poneys de polo que, dans les 24 mois suivants, avaient developpé des maladies articulaires. En conclusion, le CS du liquide synovial semble être un marqueur précoce des destructions de la cartilage articulaire.

(Traduit par les auteurs)

Introduction

Articular cartilage is a specialized connective tissue that consists essentially of chondrocytes embedded in an abundant extracellular matrix. It resists and redistributes impact loading of the joint, while providing a resilient articular surface. These properties depend on the structural organization of the extracellular matrix of macromolecules. The extracellular matrix of cartilage is composed of a dense network of collagen fibers (1) that entrap a high concentration of proteoglycans (PG) and other noncollagenous proteins.

The major noncollagenous component of cartilage matrix is the PG aggrecan (2), composed of a ~200 kDa protein core containing 3 globular domains, about 100 chondroitin sulphate (CS) chains distributed along the CS1 and CS2 domains, and about 30 keratan sulphate chains attached to a repeat domain, located just N-terminal to the CS domains. Aggrecan forms large aggregates with hyaluronic acid (HA) and link protein (3), and also interacts with other macromolecules (4). About 5% of the cartilage wet weight is aggrecan, which provides an extremely high fixed charge density that attracts and traps water, resulting in an expansion of the cartilage matrix.

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This is mainly caused by the CS chains. Tightly packed collagen fibrils resist this expansion, giving the cartilage the capacity to resist compressive forces. Changes in PG and glycosaminoglycan (GAG) structure and concentration result in changes of compressive stiffness and contribute to cartilage damage (5,6).

Articular cartilage damage, whether traumatic or inflammatory in origin, can be studied by means of the synovial fluid biomarkers in the cartilage matrix turnover and inflammatory mediators (7). Biomarkers reflecting aggrecan and collagen II turnover are capable of signaling changes in cartilage matrix homeostasis (8,9).

Prostaglandin E_2 (PGE_2) plays an intimate role in articular inflammatory and nociceptive pathways. Its release from both synovial cells and chondrocytes is stimulated by joint inflammation, injury, vascular distension, and stress (10,11). Prostaglandin E_2 is considered a sensitive predictor of joint disease (12), and its concentration of synovial fluid is high in most (if not all) joint diseases, including osteoarthritis (OA). The rise in PGE_2 concentration is very rapid, peaking 2 to 9 h after the inflammatory stimulus (13,14). In horses, although PGE_2 concentration was not correlated to the radiological signs of any joint disease (15), it is well correlated with lameness (16).

Polo is an equestrian team sport that demands quick bursts of speed, followed by a rapid turn and stop, in pursuit of the ball through its many movements. The mounts used for polo are traditionally called "polo ponies," in reference to their agility. A polo match is divided into time periods named "chukka" or "chukkers," lasting about 7 min each, with the horse being on the move throughout. So, polo ponies require considerable training and ongoing conditioning due to the extreme demands placed on them.

A young horse that has not yet started any polo training is called a "polo prospect." A young horse that is in training to become a polo pony is often referred to as a "green horse." Young polo prospects go through years of specialized training before they become what is called a "made pony," one that is ready for use in tournament polo.

The most common sources of lameness in polo ponies are tendonitis of the superficial digital flexor tendon and OA of the metacarpophalangeal joint. Furthermore, OA of the metacarpophalangeal joint is the most common articular problem leading to early retirement. The aim of this study was to investigate possible changes in synovial fluid biomarkers within the polo season in polo prospects, green horses, and high handicap made ponies that could indicate horses susceptible to OA.

Materials and methods

Materials

The following materials were obtained and used throughout our study. Standard GAG chondroitin 4-sulphate (from whale cartilage), dermatan sulphate (from hog skin), and HA (from umbilical cord), and *Streptomyces hyalurolyticus* hyaluronate lyase (EC 4.2.2.1, HA lyase, or hyaluronidase) were obtained (Sigma-Aldrich, St. Louis, Missouri, USA). Heparan sulphate (from bovine pancreas) and chondroitin AC lyase (EC 4.2.2.5, from *Flavobacterium heparinum*) were prepared by methods previously described (17,18). Agarose (standard, low M_r) was obtained (Bio-Rad Laboratories, Hercules, California, USA).

Table I. Joints, gender, age, time of training, and groups of polo ponies

Animal	Joints	Gender	Age (years)	Training (years)	Group
1	R and L	Female	3	0	1
2	R and L	Female	4	0	1
3	R and L	Female	4	0	1
4	R and L	Female	3	0	1
5	R and L	Female	4	0	1
6	R and L	Female	3	0	1
7	R and L	Male	4	1–2	2
8	R and L	Female	4	1–2	2
9	R and L	Female	4	1–2	2
10	R and L	Female	4	1–2	2
11	R and L	Female	4	1–2	2
12	R and L	Male	3	1–2	2
21	R and L	Female	11	> 5	3
22	R and L	Female	16	> 5	3
23	R and L	Female	14	> 5	3
24	R and L	Female	14	> 5	3
26	R and L	Female	11	> 5	3
27	R and L	Female	10	> 5	3
28	R and L	Male	12	> 5	3
210	R and L	Female	10	> 5	3
I	R and L	Female	10	> 5	24 h
II	R and L	Female	11	> 5	24 h
III	R and L	Female	10	> 5	24 h
IV	R and L	Female	12	> 5	24 h
V	R and L	Female	9	> 5	24 h

Animals and sample collection

The present work was approved by the Ethical Committees Universidade Federal de São Paulo — UNIFESP (CEP 0800/07) and Universidade de São Paulo — USP (1238/2007), and was carried out in accordance with UNIFESP and USP guidelines, and also in accordance with EC Directive 86/609/EEC for animal experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm).

A total of 50 healthy joints in 25 polo ponies, classified into 4 groups, were studied (Table I). Only horses that were free from lameness and with normal metacarpophalangeal joints (by ultrasound and radiography) were included. These animals were submitted to clinical examinations of both forelimbs every 28 d, during the whole experimental period (320 d). These examinations included lameness grade (0 to 5), flexion test, and effusion grade of metacarpophalangeal joints. Furthermore, ultrasonographic and radiographic evaluations of both metacarpophalangeal joints were done on days 40, 80, 240, and 320 of the polo season. Clinical and radiographic examinations were repeated 24 mo after the end of the experimental period. Detailed results are presented elsewhere (19)

To investigate the acute effect of exercises, synovial fluid samples (0.5 mL) were aspirated directly (neat) and without lavage from 10 metacarpophalangeal joints of 5 made polo ponies (numbers I to V, Table I), 9 to 12 y old, high handicap, all submitted to the same training scheme, 24 h before the polo match, and 3 h, 6 h, and 24 h after the end of the match.

To analyze the possible changes in synovial fluid during the polo season (320 d), horses were classified into 3 groups: group 1 — polo prospects, 6 polo ponies, 3 to 4 y old, who were beginning the polo training with no previous participation in tournaments (12 joints); group 2 — green horses, 6 polo ponies, 3 to 4 y old, who were under specialized training for at least 1 y (12 joints); and group 3 — high handicap made ponies, 8 polo ponies, 10 to 16 y old, who had been participating in tournaments for at least 5 y. Each group was submitted to a training scheme reflective of its respective skill level. This consisted of 30 min trot or canter and 1 h walk 5 d per wk with sporadic game training for group 1, and 40 min trot or canter and 1 h walk 6 d per wk (except on game days), with either game training or polo match 2 to 3 times per wk, for groups 2 and 3.

Synovial fluid samples (2 to 5 mL) were aspirated directly (neat) from metacarpophalangeal joints (left and right) at the end of tournout (a period of rest or vacations for polo ponies prior to a polo season, basal levels), and on days 40, 80, 240, and 320 of the polo season, always 24 h after the end of a polo match.

Synovial fluid cell count, soluble protein, PGE_2, and urea concentrations

For nucleated cell count, 1 mL of each synovial fluid sample was centrifuged at $100 \times g$ for 10 min. The supernatant was removed, and the pellet resuspended in 1 mL of phosphate-buffered saline (PBS) solution. The cells were counted in a Neubauer counting chamber, and the differential cell count was determined in smears stained using May Grunwald-Giemsa.

The supernatant was divided in aliquots that were used to measure soluble proteins, PGE_2, urea, and GAG. Soluble proteins were measured in 4 µL aliquots by biuret reaction in an automated biochemical analyzer (Labmax 240; Tokyo Boeki Machinery Ltd., Tokyo, Japan). The PGE_2 was quantified by an enzyme immunoassay kit (Cat #514010; Cayman Chemical, Ann Arbor, Michigan, USA) (20). Urea was determined in synovial fluid samples (5 µL) by completely automated enzymatic reactions (urease-glutamate dehydrogenase; Randox RX, Crumlin, County Antrim, United Kingdom). Urea concentration was used to correct the protein, PGE_2 and GAG concentrations for fluid volume (21), because urea is a robust indicator of correction for dilution of synovial fluid volume due to joint effusion (22).

Glycosaminoglycans: HA and CS

A simple and reliable method was used for the identification and quantification of synovial fluid GAG. This procedure was based on: i) migration of GAG in agarose gel electrophoresis in a special buffer system (PDA), that allows the complete segregation of CS and HA; ii) differential staining of sulphated and non-sulphated GAG by using toluidine blue in different pH levels; and iii) susceptibility to specific GAG lyases. This procedure also avoids interference from other macromolecular and ionic components.

Synovial fluid supernatant samples (100 µL) were submitted to proteolysis (4 mg/mL maxatase, detergent alkaline family of serine endopeptidases isolated from *Bacillus subtilis*, EC 3.4.21.62, in 0.05 M Tris-HCl, pH 8.0, 200 µL). After overnight incubation at 50°C, maxatase was heat inactivated (15 min, 100°C), and debris was removed by centrifugation ($3000 \times g$, 10 min, room temperature). The supernatant was freeze-dried and resuspended in 50 µL of water.

Aliquots (5 µL) were submitted to 0.5% agarose gel electrophoresis in 0.05 M 1,3-diaminopropane-acetate buffer, pH 9 (PDA), as previously described (23). This is the optimum condition to separate HA from sulphated GAG. After fixation with 0.1% cetyltrimethylammonium bromide, sulphated GAG were stained by toluidine blue in an acid solution (0.1% toluidine blue in 1% acetic acid and 50% ethanol, 15 min, room temperature). The excess dye was removed with 1% acetic acid in 50% ethanol solution. The gel slab was dried at room temperature, and then non-sulphated GAG (especially HA) were stained in the same slabs by toluidine blue at pH 5.0 (0.1% toluidine blue in 0.025M sodium acetate buffer, pH 5.0). The excess dye was removed using the same buffer. The synovial fluid GAGs were quantified by densitometry of the gel slabs using a densitometer (CS-9000 Shimadzu; Shimadzu, Kyoto, Japan).

These compounds were further characterized by enzymatic degradation using specific GAG lyases: *F. heparinum* chondroitin AC lyase (24) and *Streptomyces hyalurolyticus* HA lyase (25), as described (9).

Statistical analysis

Data were evaluated for normality using the Kolmogorov-Smirnov test, which revealed that the sample distributions were parametric. Afterwards, the unpaired *t*-test was used to compare each point to the basal levels (B) of each group. Differences between groups were analyzed by analysis of variance (ANOVA). Software (GraphPad Instat 3; GraphPad Software, San Diego, California, USA) was used to do the statistical analysis. Results are presented as mean ± standard deviation (SD). Values of $P < 0.05$ were considered significant.

Results

Acute effect of polo match upon synovial fluid components

Protein, PGE_2, urea, and GAG were measured in synovial fluid samples obtained from 10 metacarpophalangeal joints from 5 polo ponies, high handicap, 24 h before (B = basal levels), and 3 h, 6 h, and 24 h after the end of a polo match. The urea concentration did not vary significantly (Figure 1). This concentration was used to correct protein, PGE_2, and GAG for fluid volume. A transitory increase in protein and PGE_2 was observed 3 h and 6 h after the end of the polo match, returning to basal levels at 24 h (Figure 1).

A representative agarose gel electrophoresis of synovial fluid GAG from ponies I through V is shown in Figure 2A, and also from 3 other polo ponies (numbers 1, 7, and 21). One sharp band, migrating as CS, and a broad band, migrating as HA, are clearly visible in all samples. These compounds were further characterized by incubation with specific lyases, and quantified by densitometry of the agarose gel slabs. The results, expressed both as concentration (mg/mL) and as GAG/urea ratios, are shown in Figure 2B. The GAG concentration did not significantly vary within the 24 h period after a polo match.

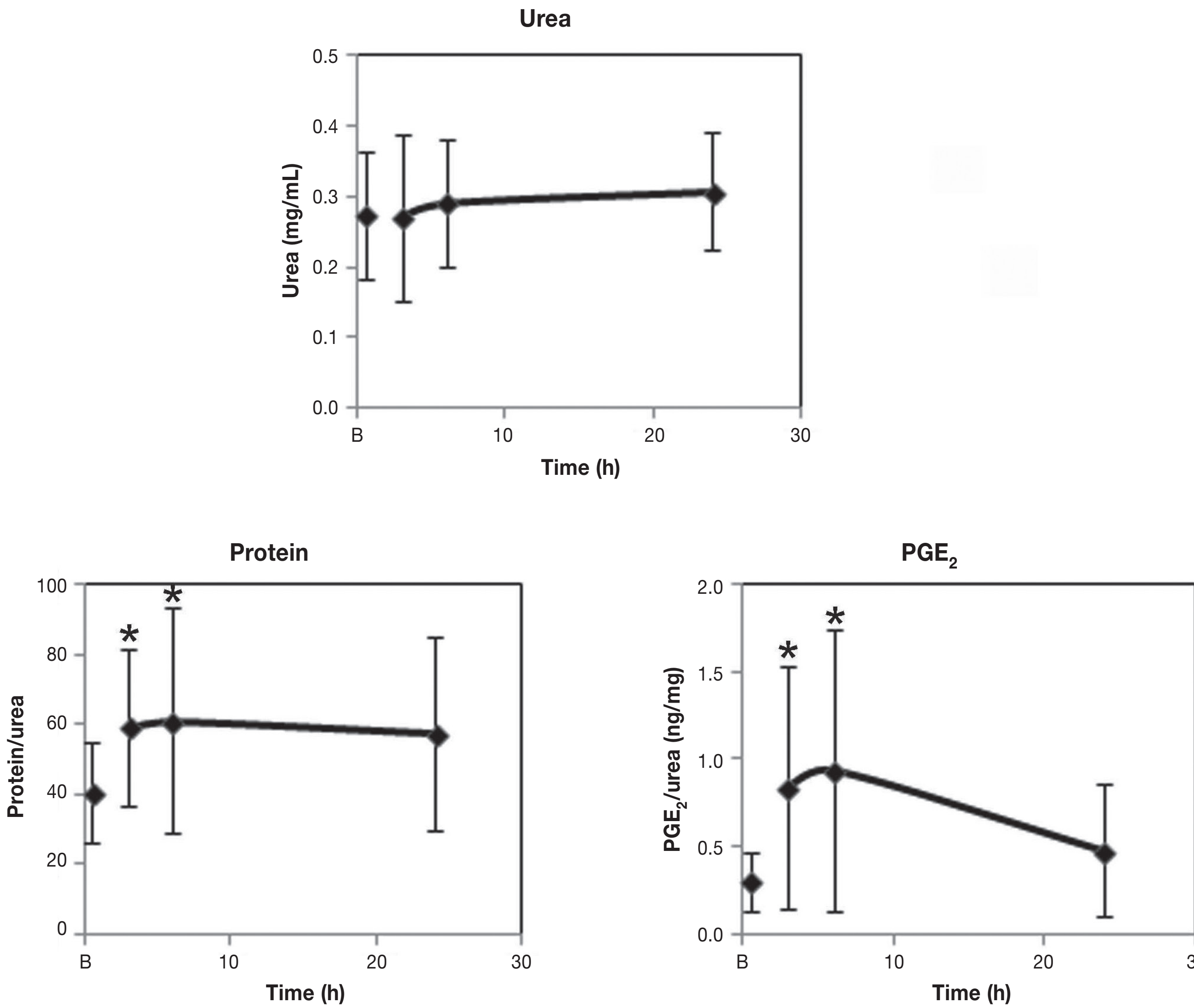

Figure 1. Urea, soluble protein, and prostaglandin E_2 (PGE_2) concentrations in synovial fluid samples of high handicap polo ponies, collected 24 h before a polo match (B = basal levels) and 3 h, 6 h, and 24 h after the end of the match. Protein and PGE_2 are presented as urea ratios, to correct for fluid volume (mean ± SD). *Statistically significant in comparison to basal levels, $P < 0.05$.

Changes in synovial fluid components during the polo season

In order to investigate possible changes during the polo season, synovial fluid samples were collected before the beginning of polo season (0 = basal), and on days 40, 80, 240, and 320, always 24 h after a polo match, from polo prospects (group 1), green horses (group 2), and high handicap made ponies (group 3). The nucleated cell count was maintained in the normal range for all groups (Figure 3). Mononuclear cells were always the most abundant cells. These data did not show any inflammatory reaction.

This finding is in agreement with the protein and PGE_2 concentrations (Figure 4). An increase in protein concentration was observed in the first 40 to 80 d for all groups, while PGE_2 did not significantly vary, again suggesting no inflammation.

For HA, an early increase occurred (compare basal to 40 d), especially for groups 1 and 2, suggesting adaptation to the exercises (Figure 5).

Concerning CS (Figure 5), similar results were obtained for groups 2 and 3, but not for group 1. In the group 1 animals, who were beginning training, the CS concentration steadily increased up to the 240th day, and remained high until the end of the polo season (in comparison to basal levels). This result was mainly due to 3 animals (numbers 1, 2, and 4), in which the CS concentration became very high (200 to 250 μg/mL, 0.5 to 1 CS/urea ratios), in contrast to other animals of the same group (15 to 30 μg/mL, ~0.1 CS/urea ratios).

Biomarkers and joint diseases

Follow-up data of the polo ponies, obtained by interviewing the referring veterinarian, revealed that 3 horses from group 1 (numbers 1, 2, and 4), 2 from group 2 (numbers 7 and 8), and 1 from group 3 (number 27, see Table I), had developed OA in the metacarpophalangeal joints within 24 mo after the end of our study. These animals presented increased joint volume and/or history of joint lameness, associated with characteristic radiographic imaging

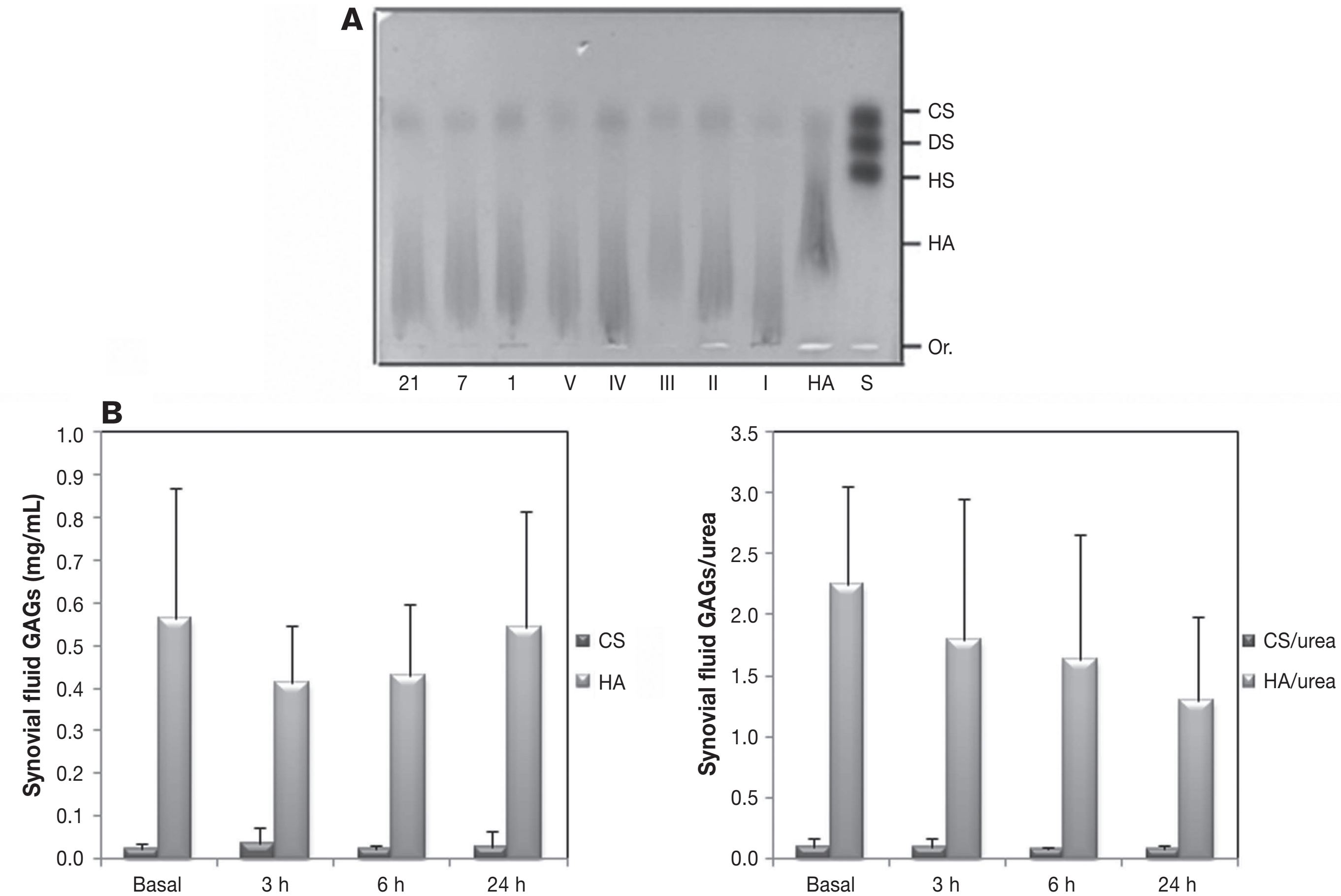

Figure 2. Synovial fluid glycosaminoglycans (GAG) from high handicap polo ponies collected 24 h before a polo match (Basal), and 3 h, 6 h, and 24 h after the end of the match. Sulphated and nonsulphated GAG were analyzed by agarose gel electrophoresis. A — Shows a representative agarose gel electrophoresis of basal synovial fluid GAG. B — Shows quantitative results, expressed both as concentration (mg/mL) and as GAG/urea ratios (mean ± SD). I, II, III, IV, V, 1, 7, and 27 refers to the polo pony numbers. CS — chondroitin sulphate; DS — dermatan sulphate; HS — heparan sulphate; HA — hyaluronic acid; Or — origin; S — mixture of standard glycosaminoglycans.

(26). The synovial fluid CS levels of these animals on the 320th day of polo season were higher than in synovial fluid of polo ponies that remained healthy and in full training (Figure 6). In contrast, the urea, HA, protein, and PGE_2 levels did not vary significantly among animals that remained healthy and animals that later developed OA. It is important to emphasize that, during our study, no clinical, ultrasonographic, or radiographic signs of OA were observed.

Discussion

Polo was the first equestrian sport in recorded history. It originated in Persia (Iran) in the 6th century BC (27), as a training game for cavalry units. Despite the antiquity of polo playing, nowadays, the sport has spread around the world. Polo ponies are subjected to complex physical challenges and there are few studies on the physiological effects of polo specialized training upon them (28), especially on their joints. In this study, biomarkers of inflammatory response and cartilage turnover were analyzed in synovial fluid samples from the metacarpophalangeal joints of polo ponies of different skill levels. The short-term (24 h) effect of polo training was also evaluated in high handicap made ponies.

While a transitory increase in synovial fluid volume during exercise was reported by Persson (29) in horses, Kingston et al (30) showed no changes in humans. Also Frisbie et al (31) showed no significant increase in synovial fluid volume following treadmill exercise in horses, but Hardy et al (32) reported increased synovial fluid volume in inflamed joints, and urea levels can be used to estimate the changes in fluid volume due to joint effusion (21,22). In this study, we measured synovial fluid urea to correct for fluid volume. Although no significant fluctuations in the urea concentration were observed, the concentrations of protein, PGE_2, HA, and CS were presented as urea ratios.

Important inflammatory reactions do not seem to have occurred, since the total cell count and the profiles of nucleated cells within synovial fluid, as well as the protein and PGE_2 concentrations, were maintained within the normal range throughout the polo season for all groups. According to Hardy et al (14), PGE_2 is an important inflammatory mediator in joints. During the first 12 h after an injury, PGE_2, 6-keto-PGF1, tromboxane B_2, and leukotriene B_4 are present, while at 24 h only PGE_2 remains (33). In the present study, protein

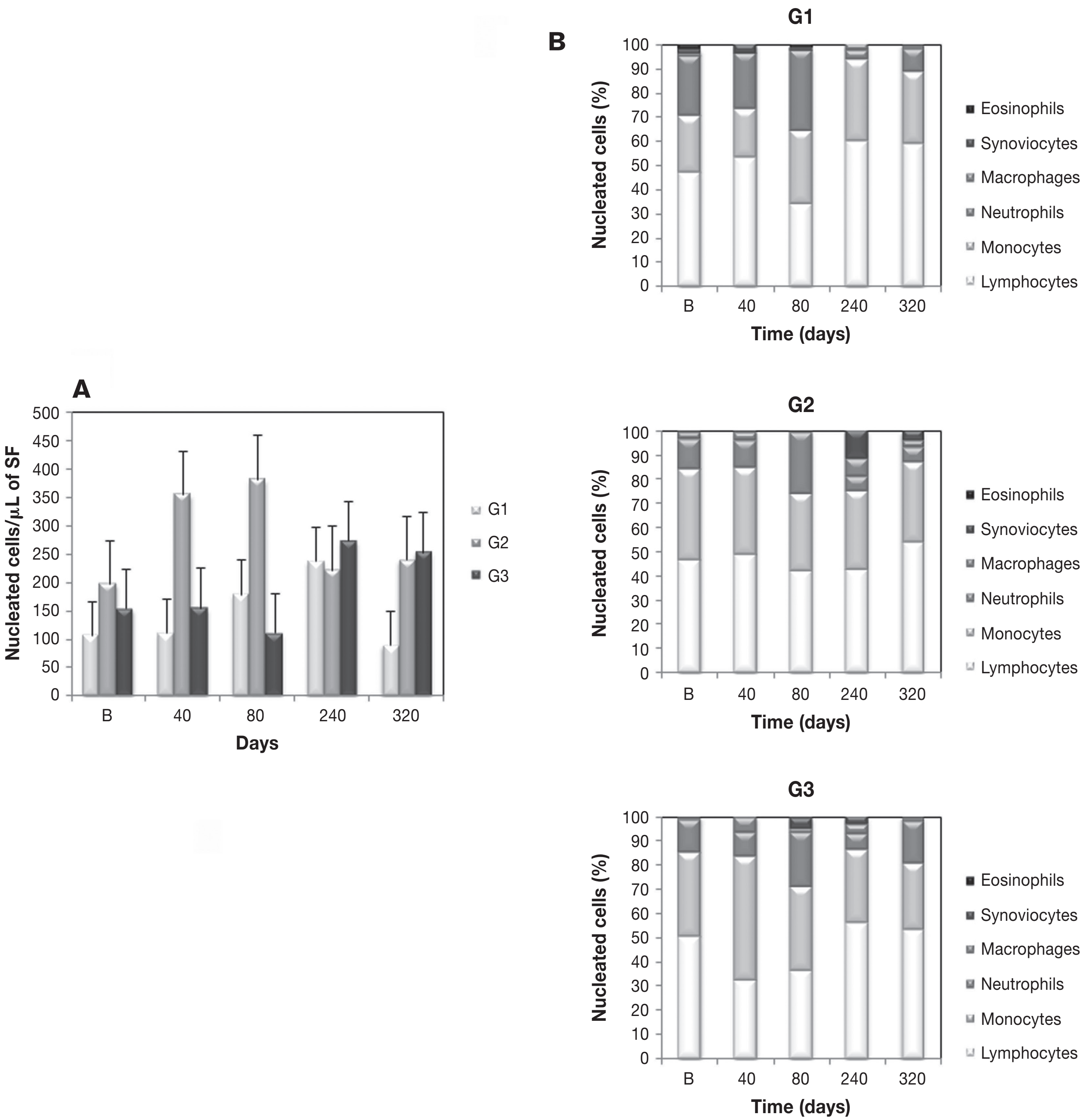

Figure 3. Nucleated cell counts of synovial fluid samples collected during the polo season from polo ponies of different skill levels. Synovial fluid samples were collected before the beginning of polo season (B, basal levels) and on days 40, 80, 240, and 320 of season from all 3 groups. A — Shows total cell count (mean ± SD). B — Relative numbers of different cell type are shown.

and PGE_2 levels were increased a few hours after the end of the polo match, but returned to basal levels within 24 h, again suggesting no inflammation.

With respect to HA, it is believed that most of the synovial fluid HA is synthesized by the fibroblast-like synoviocytes (or B-synoviocytes). Due to its high molecular weight, HA forms viscous solutions, and is largely responsible for the viscosity of the synovial fluid (34). The HA concentration increased during the first 40 to 80 d. This increase could be due to either increased synthesis or decreased degradation (or both). The HA is synthesized by HA synthases (HAS1, HAS2, and HAS3) (35). It was shown that the synthesis of HA is up-regulated in fibroblast-like synoviocytes exposed *in vitro* to CS, due to increased expression of HAS1 and HAS2, but not HAS3 (36).

The CS concentration steadily increased in polo projects (group 1), with very high values recorded in 3 horses (in comparison to basal levels). Interviewing the referring veterinarian revealed that these

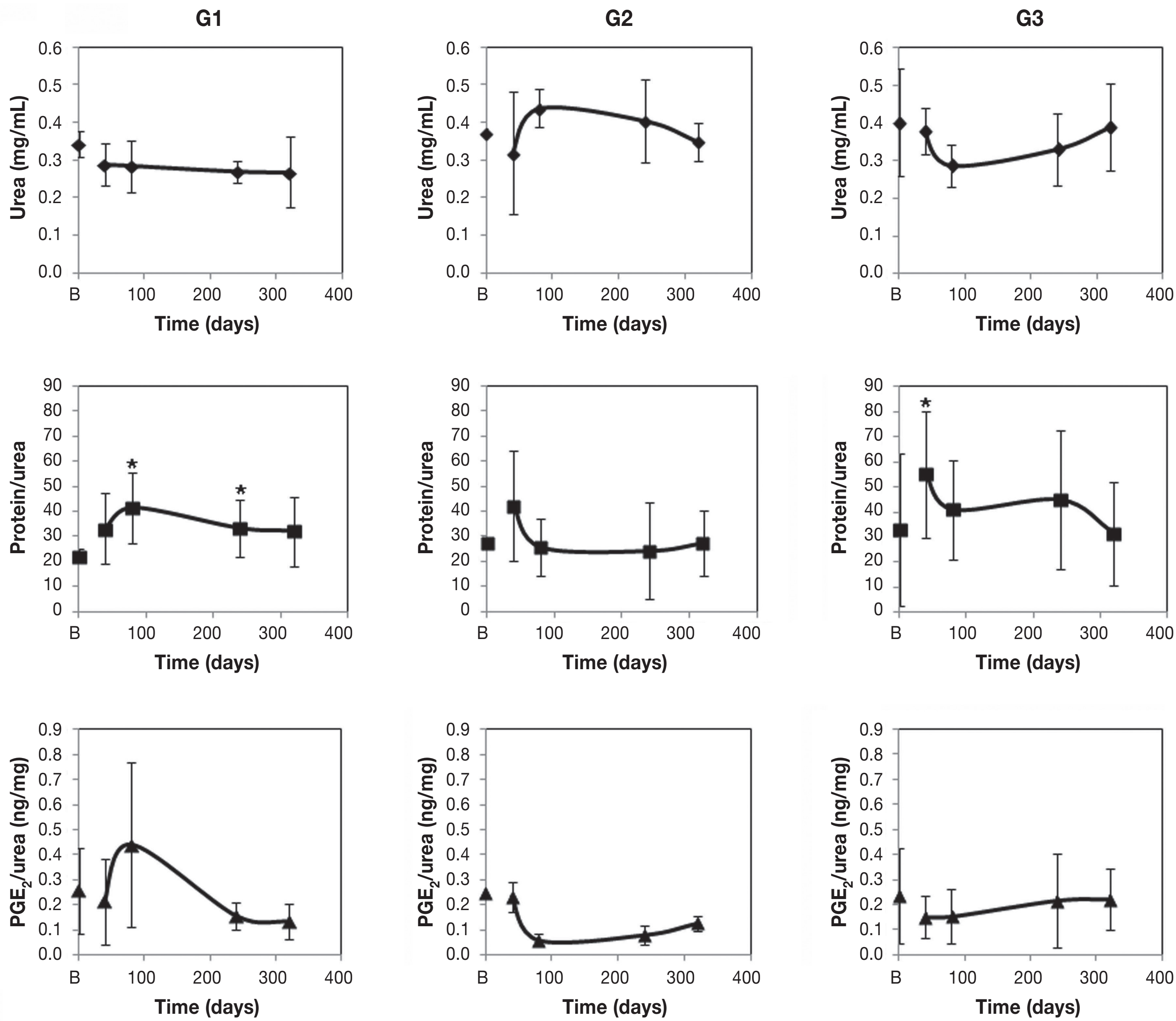

Figure 4. Synovial fluid urea, soluble protein, and prostaglandin E_2 (PGE_2) concentrations in polo ponies of different skill levels during the polo season. *Statistically significant in comparison to basal levels, $P < 0.05$.

animals, and also 2 ponies from group 2 and 1 from group 3, had developed OA in the metacarpophalangeal joints within 24 mo of the end of our study, confirmed by clinical and radiographic examinations. It is important to note that these animals did not present any signs of disease during our study.

Increased synovial fluid GAG has been previously reported in chronic and acute joint diseases (37), while contradictory results were obtained by different authors regarding exercise (31,38). However, in these studies GAG were measured by using either alcian blue or 1,9-dimethylmethylene blue (DMMB) dye binding assays, which are not specific and does not allow for the identification of CS in a mixture of GAG (39). We have previously shown that DMMB assay is inappropriately used to quantify the synovial fluid GAG because HA interferes with CS quantification (9). We have also shown that synovial fluid CS indicates abnormal joint metabolism in osteochondritis dissecans, since it is increased both in asymptomatic and in symptomatic joints (9). Therefore, CS seems to be a good biomarker to evaluate the cartilage turnover, although the analysis of aggrecan biomarkers, such as alanine-arginine-glycine-serine (ARGS)-aggrecan (31), could also give clues concerning the origin of synovial fluid CS.

Our data have shown that synovial fluid CS seems to be a useful biomarker to evaluate cartilage turnover and homeostasis. In contrast, protein and PGE_2 are useful to evaluate short-term changes, since their concentration increased a few hours after the game and returned to basal levels within 24 h.

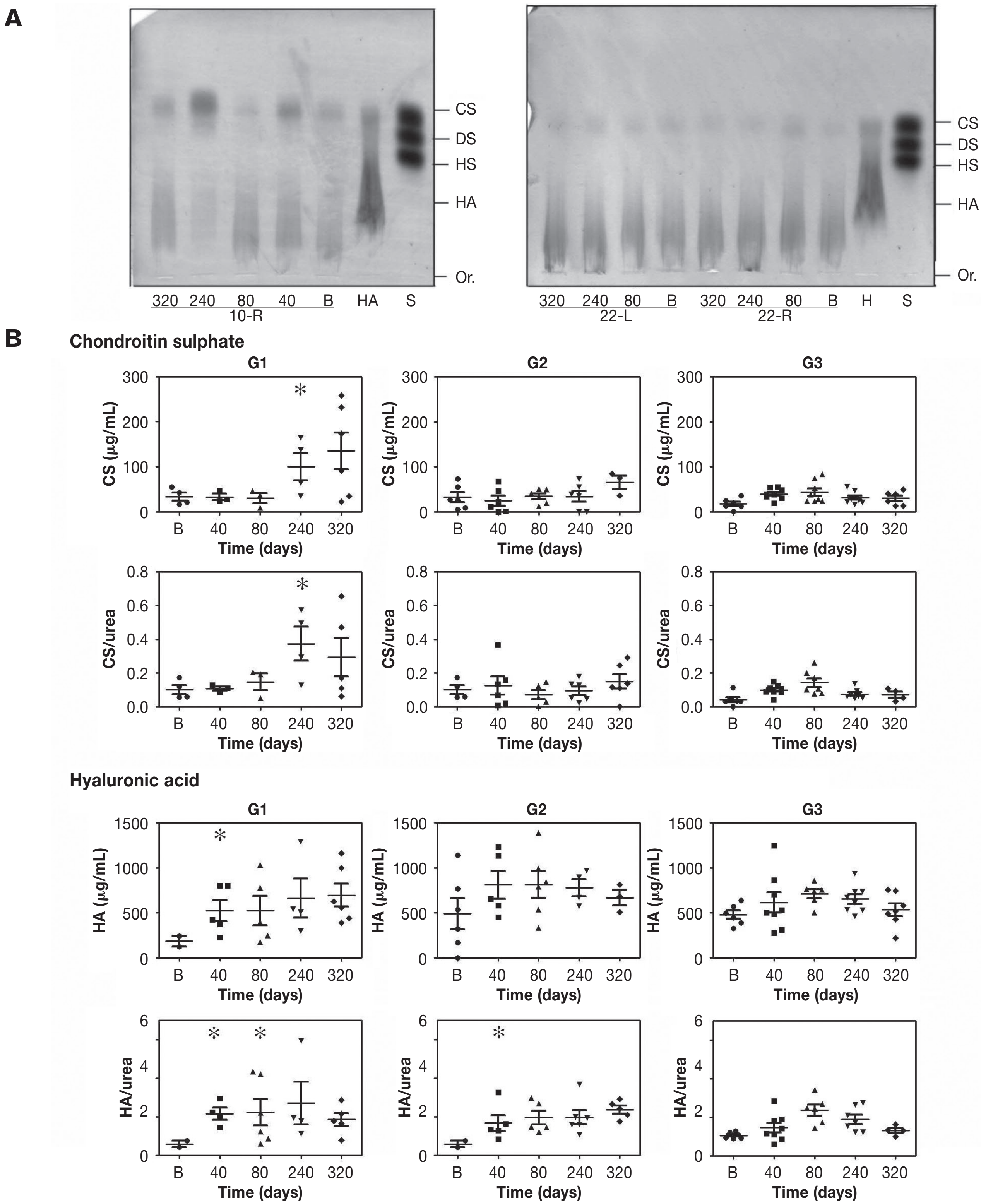

Figure 5. Glycosaminoglycans (GAG) in synovial fluid samples collected during the polo season from polo ponies of different skill levels. A — Representative agarose gel electrophoresis of samples obtained on days 40, 80, 240 and 320 and also before the polo season (B, basal) from polo ponies #10 (right metacarpophalangeal joint) and #22 (right and left metacarpophalangeal joints) are shown. Samples (100 μL) of the synovial fluid samples described in Figure 4 were submitted to proteolysis, and aliquots (5 μL) were analyzed for sulphated and non-sulphated GAGs by agarose gel electrophoresis, as described in Methods. S — mixture of standard glycosaminoglycans; DS — dermatan sulphate; HS — heparan sulphate; Or — Origin. Individual data for B — Individual data for chondroitin sulphate (CS) and (C) hyaluronic acid (HA) concentrations (expressed both as μg/mL and CS/urea and HA/urea ratios) in synovial fluid of ponies during the polo season of Polo Projects (G1), Green Horses (G2) and high handicap Made Ponies (G3) during the polo season. Horizontal lines indicate mean ± standard deviation (SD). *Statistically significant in comparison to basal (B) levels, $P < 0.05$.

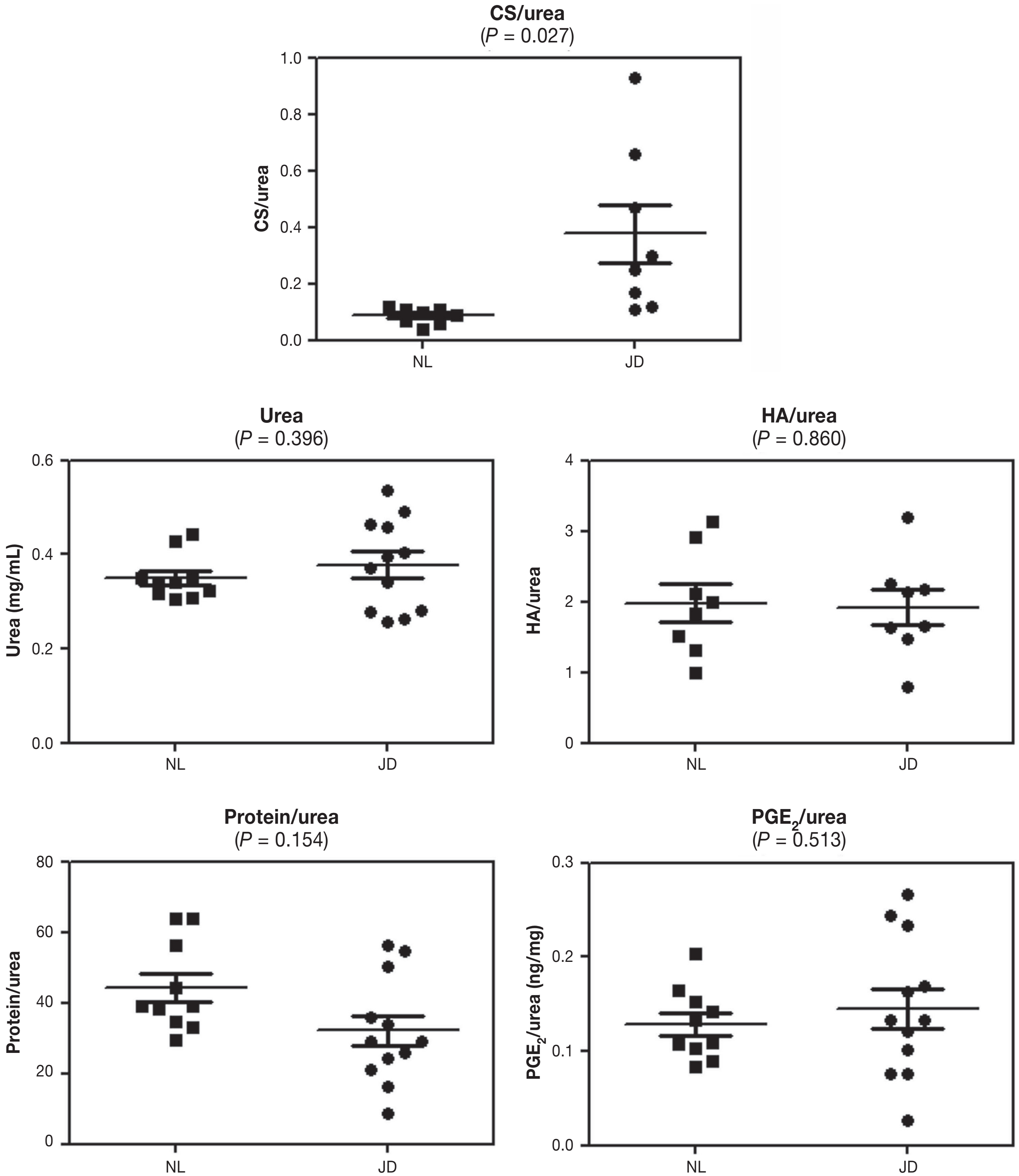

Figure 6. Synovial fluid chondroitin sulphate (CS), urea, hyaluronic acid (HA), protein, and prostaglandin E_2 (PGE_2) in polo ponies that developed osteoarthritis (OA) in the metacarpophalangeal joints within 24 mo after our study ended, in comparison to those that remained healthy. Individual values of synovial fluid urea (day 320, mg/mL), CS, HA, protein and PGE_2 (urea ratios to correct for fluid volume) in polo ponies that developed OA within 24 months after the end of our study (OA), in comparison to polo ponies that remained healthy at the end of this period (NL). Each point is the mean of 3 measurements. Horizontal lines indicate mean ± SD of all points. *Statistically significant if $P < 0.05$. NL — synovial fluid from normal, healthy joints; JD — synovial fluid from joints that developed osteoarthritis.

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